Novel Conformation-Specific Antibodies Against Matrix γ-Carboxyglutamic Acid (Gla) Protein

Undercarboxylated Matrix Gla Protein as Marker for Vascular Calcification

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Objective— Matrix γ-carboxyglutamic acid (Gla) protein (MGP), a vitamin K–dependent protein, is a potent in vivo inhibitor of arterial calcification. We hypothesized that low endogenous production of MGP and impaired carboxylation of MGP may contribute to the development or the progression of vascular disease.

Methods and Results—Novel conformation-specific antibodies against MGP were used for immunohistochemistry of healthy and sclerotic arteries. In healthy arteries, MGP was mainly displayed around the elastin fibers in the tunica media. The staining colocalized with that for carboxylated MGP, whereas undercarboxylated MGP (ucMGP) was not detected. In atherosclerotic arteries, ucMGP was found in the intima, where it was associated with vesicular structures. In Mönckeberg's sclerosis of the media, ucMGP was localized around all areas of calcification. The results indicate that ucMGP is strongly associated with vascular calcification of different etiologies. In a separate study, serum MGP concentrations in a cohort of 172 subjects who had undergone percutaneous coronary intervention were significantly reduced compared with an apparently healthy population.

Conclusions—These data show that impaired carboxylation of MGP is associated with intimal and medial vascular calcification and suggest the essentiality of the vitamin K modification to the function of MGP as an inhibitor of ectopic calcification. **(*Arterioscler Thromb Vasc Biol.* 2005;25:1629-1633.)**

Key Words: matrix Gla protein (MGP) ■ vitamin K ■ calcification ■ atherosclerosis

The extracellular fluids in the human body contain calcium and phosphate in high concentrations, even exceeding the solubility product for spontaneous precipitation.[1] However, physiological calcification is restricted to bone and teeth, whereas soft tissue calcification is regarded as pathological. Vascular calcification can occur at 3 anatomic sites: the intima where it is associated with atherosclerosis, the tunica media, and the heart valves. Huang et al showed that coronary artery calcification does not significantly affect stability of atheroma,[2] and the involvement of calcium salt accretion on cardiovascular disease is not known yet. However, overall vascular calcification is regarded as one of the major complications of cardiovascular disease and is an independent risk factor for myocardial infarction (MI) and cardiac death.[3–6] Therefore, prevention of vascular calcification is a prerequisite for human health. Inhibition of calcification is regarded presently as an active process in which a variety of proteins are involved throughout the body.[7] In the vasculature, a major calcification inhibitory factor is matrix γ-carboxyglutamic acid (Gla) protein (MGP), a vitamin K–dependent protein synthesized by vascular smooth muscle cells (VSMCs).[8,9] Its 5 Gla residues are formed in a post-translational carboxylation reaction in which vitamin K functions as an essential cofactor.[10,11] MGP in its carboxylated form will be designated here as GlaMGP. The presence of the Gla residues is critical for MGP function, and undercarboxylated, inactive species of MGP (designated as GluMGP) are formed during inadequate vitamin K status or as a result of vitamin K antagonists. In animal models, it was demonstrated that impaired MGP synthesis[12,13] as well as treatment with vitamin K antagonists[14] result in arterial calcification within 2 to 4 weeks, whereas in humans, it was demonstrated that oral anticoagulant treatment is associated with substantially increased heart valve calcification.[15] Human MGP promoter polymorphisms were identified and demonstrated to be associated with low MGP expression and low serum MGP

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levels.[16] In addition to animal models for impaired MGP expression, polymorphisms in humans were demonstrated to be associated with an increased risk for MI in humans.[17]

Calcification of vascular tissue has been observed in a number of conditions, including aging, diabetes, and renal disease.[18] Arterial calcification is associated with MI and ischemia in peripheral vascular disease, especially in diabetes and end-stage renal disease. Arterial calcification can occur in the intima (atherosclerosis), where it is associated with macrophage- and lipid-rich atherosclerotic lesions; it may also occur in the media (Mönckeberg's sclerosis), independently of atherosclerosis and almost exclusively associated with VSMCs.[19] The independence and unique features of these conditions is suggestive of different etiologic pathways. Mönckeberg's sclerosis gained increasing attention because of findings linking it with mortality, cardiovascular disease in diabetes, advanced renal disease, lower extremity amputation, and poor outcomes in peripheral arterial disease.[4]

Using conformation-specific antibodies, Sweatt et al reported recently the presence of poorly carboxylated MGP in the calcified vasculature of aging rats.[20] In another study, Price et al induced arterial calcification in rats by vitamin D plus warfarin treatment and demonstrated by protein sequencing that the MGP that accumulated at the sites of calcification was poorly carboxylated.[21] Because only carboxylated MGP has calcification-inhibitory activity, we hypothesized that there are 2 independent conditions contributing to the development or progression of cardiovascular calcification associated with MGP: low constitutive MGP synthesis in the vasculature and incomplete MGP carboxylation as a result of poor vitamin K status. Phase I of the present study was performed in surgical specimens obtained from our department of pathology. These specimens were used to study the localization and carboxylation status of MGP using a panel of novel monoclonal antibodies. Study phase II was facilitated by our access to a study in which the effects of exhaustion on angioplasty patients were evaluated on new coronary events.[22] In this cohort, we tested this hypothesis by measuring MGP in serum from healthy subjects and cardiovascular patients.

Materials and Methods

Materials and Methods can be found in the online supplement, available at http://atvb.ahajournals.org.

Results

Study Phase I: Measurement of MGP Species in Tissues

Atherosclerosis

Sections of carotid arteries with various degrees of atherosclerosis from 5 different donors were compared with normal, nonaffected carotid arteries (also 5 donors; Table I, available online at http://atvb.ahajournals.org). In normal vascular tissue, neither calcification nor lipid infiltration was observed. Staining with CD68 for macrophage infiltration was negative (data not shown). In Figure 1, immunohistochemical localization of MGP species during different stages of atherosclerosis is shown. In all healthy arteries (Figure 1), total MGP (tMGP) was not well visible in the intima, but most of it had accumulated around the medial elastin fibers (Figure 1A); GluMGP was not found at this site (Figure 1B). With the antibody specifically recognizing GlaMGP (Figure 1C), we found staining coinciding with tMGP, suggesting that the elastin-associated MGP in the healthy vasculature mainly occurs in its carboxylated form. In early atherosclerotic lesions (Figure 2; stage II to III), substantial intima thickening was observed; calcification was seen occasionally as isolated stippling, and lipid accumulation was evident from oil red-O staining (data not shown). Also, CD68 staining was positive, demonstrating macrophage infiltration. In these preparations

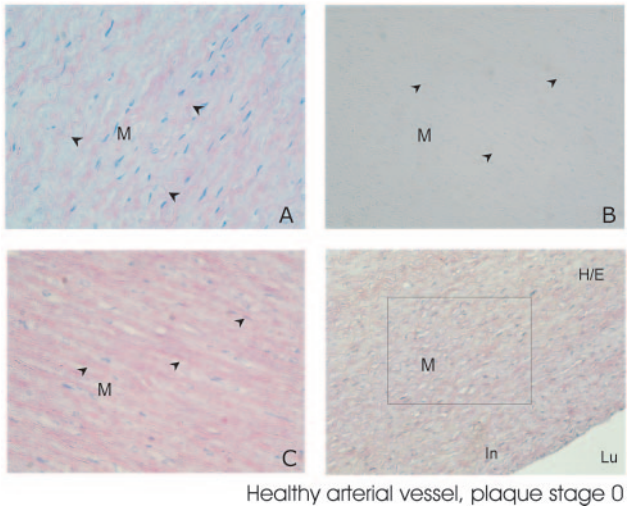

Figure 1. Immunohistochemical localization of MGP species in an apparently healthy arterial vessel wall. A represents total MGP; B, GluMGP; and C, GlaMGP staining. The GluMGP protein seems absent at this stage, whereas total MGP and GlaMGP are present. Arrows indicate the elastin fibers in the media. M indicates media; In, intima; Lu, lumen. The hematoxylin/eosin (H/E) staining demonstrates no signs of abnormalities.

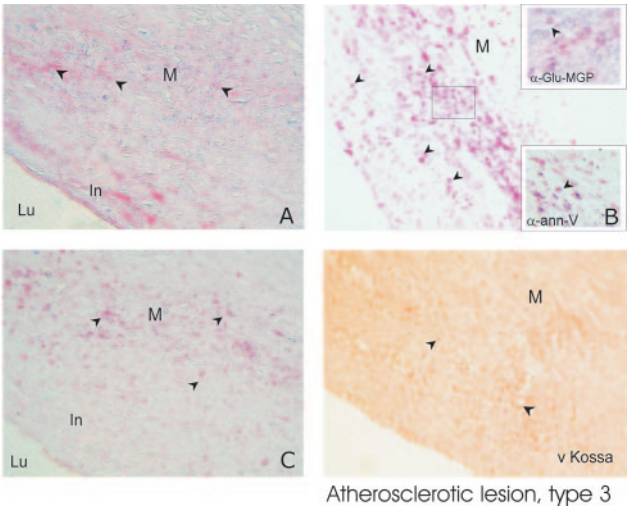

Figure 2. Immunohistochemical localization of MGP species in stage II to III atherosclerotic lesion. A represents total MGP; B, GluMGP; and C, GlaMGP staining. The predominant form is GluMGP, mainly around vesicular structures. The von Kossa stain reveals that these vesicular structures contain calcium. The inset in B demonstrates that the rounded structures are annexin V positive, thus, they most likely expose PS. Arrows indicate vesicular structures (size ≈30 to 850 nm). M indicates media; In, intima; Lu, lumen.

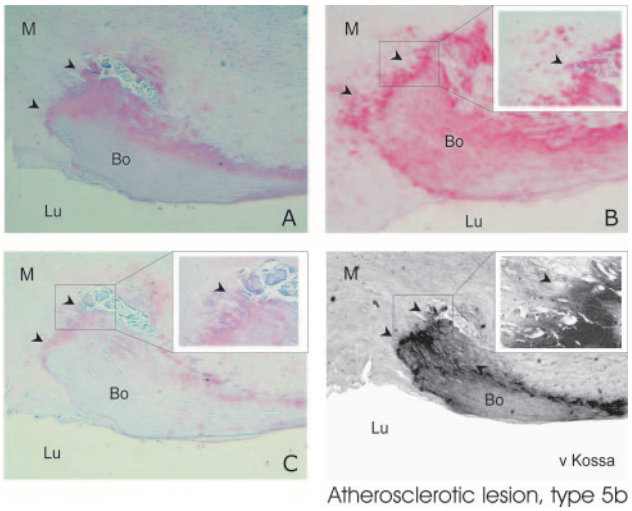

Figure 3. Immunohistochemical staining of MGP species in stage Vb atherosclerotic lesion. A represents total MGP; B, GluMGP; and C, GlaMGP staining. GluMGP staining is the predominant staining, colocalizing with von Kossa staining. In the insets can be seen that at the interface of calcium crystal (Bo) and surrounding tissue, vesicular structures are present. These rounded structures are heavily calcified (von Kossa staining). Arrows indicate vesicular structures. M indicates media; Lu, lumen; Bo, bone.

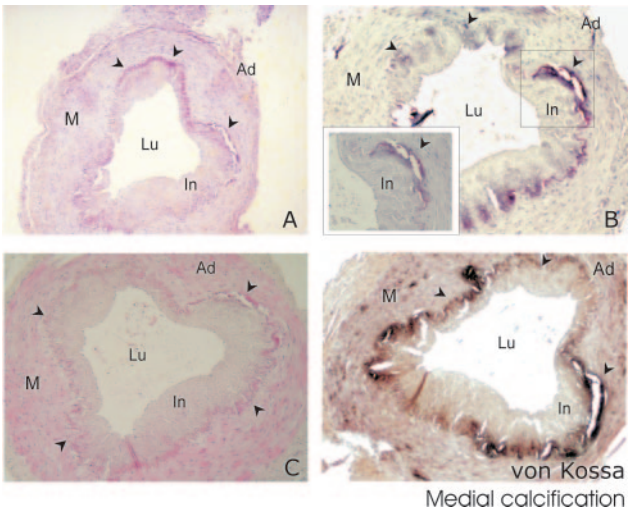

Figure 4. Immunohistochemical localization of MGP species in Mönckeberg's sclerosis of the media. Four subsequent sections from a peripheral diabetic artery were stained in different ways. Because the sections are slightly tilted from left to right, the massive calcified area at top left to middle may be used for orientation. A represents total MGP; B, GluMGP; and C, GlaMGP staining. It can be seen clearly from these pictures that Glu–MGP completely colocalizes where calcification is present. Arrows indicate calcified areas of the elastic lamina. M, indicates media; In, intima; Lu, lumen; Ad=adventitia.

(Figure 2), a different distribution pattern of MGP was observed: staining for tMGP showed MGP accumulation in vesicular structures (Figure 2A and 2D) in the intima and similar patterns for GluMGP (Figure 2B). However, staining for GlaMGP was poor (Figure 2C). Moreover, von Kossa staining for calcification revealed small calcified spots (arrows). The diameter of these rounded structures was estimated at 30 to 850 nm, which is similar to that of apoptotic bodies, lipid debris, or cellular remnants. The mean vesicular size±SD was 246±13 nm. The size was measured using a microscope coupled to a computerized morphometry system (Quantimed 570; Leica; data not shown). More evidence that the vesicular structures were of cellular origin came from staining with annexin V (Figure 2B, inset). Annexin V is known to bind phosphatidyl serine (PS)–exposing cells or apoptotic (PS positive) bodies. Together, these data suggest substantial undercarboxylation of MGP in vesicular structures formed during the progression of atherosclerosis. In type Vb plaques (characterized by calcium salt precipitates and bone formation; Figure 3), calcification was demonstrated by von Kossa staining. A positive staining for apoptosis was observed at the interface between tissue and calcium crystals. At this stage, substantial amounts of MGP had accumulated around the mineralized areas, and comparable intensities for tMGP (Figure 3A) and GluMGP (Figure 3B) staining were observed. The staining for GlaMGP was poor at this stage, suggesting that most of the MGP was present in its undercarboxylated nonactive form (Figure 3C). Also, at the interface of calcium crystal and surrounding tissues, vesicular structures were present, mainly colocalizing with GluMGP (Figure 3B and von Kossa, inset).

Mönckeberg's Sclerosis

To investigate MGP localization in medial calcification, we stained for different conformations of MGP in peripheral arteries (n=6; Table I). Staining with oil red-O and CD68 antibodies confirmed the absence of inflammation and lipid infiltration (data not shown). Figure 4 shows MGP localization in an example of a peripheral artery from a diabetic patient. This is a typical form of media sclerosis (Mönckeberg's sclerosis) with major calcifications starting around the elastin fibers in the tunica media (von Kossa). Most tMGP was localized in the noncalcified areas (Figure 4A), whereas GluMGP was almost exclusively found to be associated with areas of calcification (Figure 4B). More advanced stages of media calcification were still devoid of lipids and macrophages but contained the vesicular GluMGP-rich structures as observed in atherosclerosis. GlaMGP was also found around the elastic fibers, although less specific associated with calcified areas (Figure 4C). Together, the immunohistochemical data demonstrate that in atherosclerotic intima sclerosis and diabetic media sclerosis, GluMGP is abundantly present, suggesting local vitamin K deficiency and impaired protection against calcification attributable to poor MGP carboxylation.

Study Phase II: MGP Measurement in Serum

Circulating MGP-related antigen and vitamin K status were measured in an apparently healthy reference population (Table IIa, available online at http://atvb.ahajournals.org) as well as in a group after percutaneous coronary intervention (PCI; Table IIb). Table IIa shows the demographic and medical characteristics of the healthy control group and Table IIb that of the patient population. Figure 5 shows the serum MGP distribution in the reference (mean ± SD; 11.5±4.0) and the patient group (5.0±2.1 and 6.23±1.9, respectively). Obviously, serum MGP was below the normal range at 1.5 and 7.5 months after PCI. The fact that it remained low even at 7.5 months after PCI is suggestive for low constitutive MGP expression, not related to the surgical intervention. In a subse-

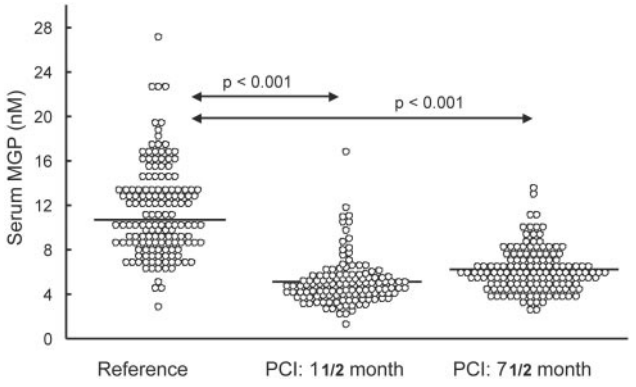

Figure 5. Serum MGP in health and disease. Left, Reference population. Middle, PCI patients at 1.5 months after surgery. Right, Same cohort at 7.5 months after surgery.

quent analysis, we tested whether the medication used might affect the circulating MGP concentration. Cross-tables with quartile distribution of MGP were compared with baseline medication, and the significance of differences between users and nonusers was tested with the Pearson χ^2 test. There was no effect of medication on the serum MGP concentration.

Discussion

In this article, we demonstrated that low circulating MGP and an impaired γ-carboxylation of the protein at its tissue site of expression are associated with the development and progression of cardiovascular disease.

In a first approach, we investigated by immunohistochemistry whether undercarboxylation (and thus suboptimal protein activity) of MGP is associated with various forms of vascular disease. To this aim, we developed conformation-specific antibodies exclusively recognizing GluMGP and GlaMGP (Figure I, available online at http://atvb.ahajournals.org). In healthy arteries, devoid of calcification and lipid or macrophage infiltration, total MGP was deposited mainly along the elastin fibers. It is noteworthy that at this stage, almost no GluMGP was found. Apparently, most of the MGP is active in the healthy vasculature, which is demonstrated by the staining with anti-GlaMGP. The abundance of active, carboxylated MGP is consistent with in vitro cell culture data, showing that the vitamin K antagonist warfarin induces calcification, whereas vitamin K has a protective effect.[28] Stage III atherosclerosis is associated with lipid infiltration and inflammation, as defined by Virmani et al.[25] Substantial amounts of MGP accumulated in vesicular structures, which may be apoptotic bodies or lipid debris originating from cell death. Staining for GluMGP revealed that a major part of MGP associated with these structures was undercarboxylated, suggesting that at these increased expression levels, the vitamin K–dependent carboxylation machinery loses its ability to γ-carboxylate all the MGP synthesized by the vessel wall. The most likely explanation for this impaired carboxylation is that there are insufficient vitamin K reserves in the vessel wall to cope with the increased MGP expression, but other explanations cannot be ruled out. The abundance of inactive undercarboxylated MGP in vesicular structures is consistent with in vitro cell culture data, showing that matrix vesicles and apoptotic bodies, which are thought to be the nucleation site for vascular calcification, have an increased tendency to calcify if they originate from cells treated with warfarin.[29] Moreover, previous studies showed that apoptosis is involved in calcification of VSMC multicellular nodules[30] and that isolated VSMC-derived apoptotic bodies and vesicles[29] could calcify in a similar manner to chondrocyte-derived matrix vesicles.[31,32] It is noteworthy that MGP is involved in calcification and apoptosis of VSMCs and chondrocytes. In stage Vb atherosclerosis, calcification and bone formation were accompanied by MGP accumulation, predominantly in the contact area between mineral and tissue; also, in this case, a substantial part of the MGP occurred in an undercarboxylated form, and the staining for carboxylated MGP in a corresponding section was poor. This is consistent with studies in young rats showing that warfarin causes vascular calcification within 2 weeks because of inactivation of MGP,[14,21] and with studies in aging rats showing that vascular calcifications are associated with undercarboxylated MGP.[20] In a second approach, we explored whether our findings in atherosclerotic lesions were also applicable to Mönckeberg's sclerosis, a form of vascular calcification of completely different etiology. In this condition, MGP was associated primarily with the elastin fibers. Most MGP was found in the noncalcified areas, whereas the GluMGP fraction was almost exclusively present in the extensively calcified parts of the tunica media. Although other explanations are possible, these data are consistent with the hypothesis that local poor vitamin K tissue status is a risk factor for vascular calcification.

Subsequently, we addressed the question whether the putative low-tissue MGP expression and poor tissue vitamin K status (as deduced from GluMGP) can be monitored in serum. A broad range for serum MGP was found in an apparently healthy reference population. Possible reasons for variation in this group include promoter polymorphisms affecting MGP expression[16,17] and subclinical levels of atherosclerosis and vascular calcification (known to be common in all adults),[33] which may induce MGP expression to variable extents. It should be noted that the reference population was not screened for vascular disease and presumably contained subjects with thus far undetected arterial calcification. About one third of the reference population had serum MGP levels >15 nmol/L, whereas in the patient group, high serum MGP levels were not present, and 90% of all values were <10 nmol/L. The median value for the reference group was substantially and significantly higher than that for the patient group at both time points measured. This is consistent with a recent study from Japan, in which an inverse correlation was observed between coronary artery calcification and circulating MGP.[34]Unfortunately, the GluMGP antibody did not lend itself for testing in serum because this antibody only recognizes ucMGP after fixation or denaturation.

One key question arising from this study is: does the measurement of serum MGP have any diagnostic utility? From the limited data presently available, it seems that the assay may be useful as an extra marker for cardiovascular risk assessment, complementary to existing tests such as serum cholesterol and blood pressure. The clinical utility of the assay in the diagnosis of cardiovascular patients remains unclear. During the development of atherosclerosis, vascular MGP synthesis may be triggered locally,[8,35] but whether this

is reflected by an increase of circulating MGP to levels above the normal range is currently unknown.

It is noteworthy that accumulation of GluMGP in calcified arteries was identified as a second variable associated with cardiovascular disease. The most probable mechanism underlying this observation is incomplete MGP carboxylation resulting in suboptimal inhibition of arterial calcification. This hypothesis is strengthened by preliminary data from our group showing that patients with severe calcifications have an overall vitamin K deficiency, as measured by the ratio of undercarboxylated osteocalcin to carboxylated osteocalcin and plasma vitamin K concentrations.

Unlike low MGP expression, incomplete MGP carboxylation may be amenable to treatment by increasing vitamin K intakes. To follow such treatment, a serum-based assay for undercarboxylated MGP is needed that would allow the assessment of the fraction of total MGP that circulates as ucMGP and the response of this fraction to increased vitamin K intakes. Such an assay would provide a better insight of the relationship of MGP activity to vitamin K status in cardiovascular disease.

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